

## ALZHEIMER'S DISEASE TREATMENT METHOD

The present invention relates to a method for treatment and/or prevention of diseases associated with the presence of amyloid deposits, which include Alzheimer's disease.

## STATE OF THE ART

Certain facts are known about the biochemical and metabolic phenomena associated with the presence of Alzheimer's Disease (AD). Two structural and histopathological changes observed in the brains of those with AD are neurofibrillar tangles (NFT) and amyloid deposits. Intraneuronal neurofibrillar tangles are also present in other neurodegenerative diseases, but the presence of amyloid deposits both in the intraneuronal spaces (neuritic plaques) and close to the microvasculature (vascular plaques) seems to be characteristic of AD. Of these, neuritic plaques seem to be the most common (Price, D.L., and co-workers, Drug Development Research (1985) 5:59-68).

The main component of these amyloid plaques is a peptide of 40-42 amino acids denominated amyloid peptide A $\beta$ 4.

The amyloid peptide A $\beta$ 4 is a polypeptide that originates from proteolysis from membrane glycoproteins denominated amyloid peptide A $\beta$ 4 precursor proteins ( $\beta$ APP). These proteins, precursors of amyloid peptide, consist of 695 to 770 amino acids, all of them being coded by the same gene.

Two main variants of amyloid peptide A $\beta$ 4 have been identified, peptide A $\beta$ 40 and A $\beta$ 42, containing 40 and 42 amino acids, respectively, which present different tissue distributions in both physiological and pathological conditions.

The variant of 42 amino acids is the predominant form in the amyloid plaques located in the brains of patients with AD.

Until present, different possible solutions have been proposed to provide a possible vaccine against AD.

In EP526511, the administration of homeopathic doses of A $\beta$  to patients with pre-established AD is proposed. However, due to the doses used, the levels of circulating endogenous A $\beta$  in plasma hardly vary, and so no therapeutic benefit is expected.

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Schenk et al., (Nature, 1999; 400: 173-177) describe immunization of transgenic mice PDAPP with A $\beta$ 42, which overexpress human mutant APP, thus preventing the formation of amyloid plaques, neuritic dystrophy and astrogliosis.

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In WO9927944 (Schenk D.), a treatment for AD is described by administration to a patient of A $\beta$ 42.

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A phase III clinical trial in 360 patients diagnosed with medium to moderate AD in 4 European countries and the United States, in which amyloid peptide A $\beta$ 42 was used as an antigen, was discontinued after encephalitis was reported in some of the patients (Scrip Daily Online, 25 Feb 2002, S007455320, The Scientist 16 [7]: 22, April 1, 2002).

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The problem of using an endogenous protein as a vaccine (or a protein present naturally in the animal that is being vaccinated), as is the case of peptide A $\beta$ 42, the organism responds by making antibodies against A $\beta$ 42 and against smaller fractions that may also have as yet unknown physiological functions, among some of the possible problems we could mention is the possible development of autoimmune diseases due to the generation of antibodies against the

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endogenous protein, difficulty in the generation of a immune response due to failure of the immune system for recognizing endogenous antigens, and possible development of an acute inflammatory response.

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The present invention is aimed at treatment of Alzheimer's disease and other amyloid diseases by administration of a peptide, of the C-terminus part of A $\beta$ , conjugated with a protein, which in a preferred embodiment of the present invention said protein is the keyhole limpet hemocyanin.

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EXPLANATION OF THE INVENTION

The present invention relates to a vaccine for the prevention and/or treatment of Alzheimer's disease and other related amyloid diseases.

5 According to a preferred embodiment of the present invention, a vaccine is provided for the prevention and/or treatment of Alzheimer's disease and other related diseases, which overcomes the disadvantages associated with using peptides, proteins or endogenous immunogens.

10 Examples of other diseases characterized by amyloid deposits are Islandic hereditary syndrome, multiple myeloma, and spongiform encephalitis, including Creutzfeldt-Jakob disease.

15 The introduction of an immune response can be active such as when an immunogen is administered to induce antibodies that react with A $\beta$  in a patient, or passive, such as when an antibody is administered that reacts by itself with A $\beta$  in a patient.

For the aims of the present invention, the following terms are defined as follows:

20 The term "related amyloid diseases" includes diseases associated with the accumulation of amyloid which can be restricted to one organ, localized amyloidosis, or spread throughout several organs, systemic amyloidosis. Secondary amyloidosis can be associated with chronic infections (such as, for example, tuberculosis) or chronic inflammation (for example, rheumatoid  
25 arthritis), familial Mediterranean fever (FMF) and other types of systemic amyloidosis found in patients in the long-term treatment of hemodialysis. Localized forms of amyloidosis include, but are not limited to, type II diabetes and any other disease related thereto, neurodegenerative diseases with scrapie, bovine spongiform encephalitis, Creutzfeldt-Jakob disease, Alzheimer's  
30 disease, cerebral amyloid angiopathy.

The term "passive immunization" is used to relate to the administration of antibodies or fragments thereof to an individual with the intention of conferring immunity on that individual.

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In the first aspect, the invention provides the use of either a peptide that acts as an immunogen or as an antibody, in the preparation of a medication for the prevention and/or treatment of a disease characterized by the accumulation of amyloid deposits. Said methods consist of the induction of an immune response  
5 against a peptide component of the amyloid deposits in the patient. Said induction could be active through administration of an immunogen or passive through administration of an antibody or an active fragment or derivative of an antibody.

10 In a preferred embodiment of the present invention, the disease is Alzheimer's disease.

The medication obtained can be used both in asymptomatic patients such as those who show symptoms of the disease.

15 In accordance with the presence of the present invention, the compositions able to provoke an immune response directed against certain components of the amyloid plaques are effective for treatment or prevention of diseases related to amyloid deposits. In particular, in accordance with an aspect of the present  
20 invention, it is possible to prevent the progress of, reduce the symptoms of and/or reduce the deposition process of amyloid in an individual, when an immunostimulatory dose of a peptide or of an antibody obtained therefrom, is administered to the patient.

25 In accordance with an aspect of the present invention, the antibodies are obtained by immunization of mammals or birds by use of a peptide conjugated to a protein as an immunogen.

30 According to a preferred embodiment of the present invention, the mammals used for immunization can be ruminants, equines, lagomorphs, carnivores, primates, or any other animal that allows adequate quantities of serum to be extracted therefore for antibody. Among the birds used for immunization, we can mention, but in no way limit to, Galliformes, Anseriformes and  
35 Columbiformes, among others.

According to a preferred embodiment of the present invention, this provides the use of a peptide conjugated to a protein that acts as an immunogen to produce antibodies able to specifically recognize any of the predominant variants of the beta amyloid peptide A $\beta$ 40 and A $\beta$ 42 in the preparation of a medicament for the prevention and/or treatment of a disease characterized by the accumulation of amyloid deposits in the brain of a patient.

According to the most preferred embodiment of the present invention, the protein used for conjugation with the peptide is keyhole limpet protein.

In accordance with an even more preferred embodiment of the present invention, the peptide is selected from a group that consists of the peptide of SEQ ID No 1, the peptide of SEQ ID No 2, the peptide of SEQ ID No 3, the peptide of SEQ ID No 4, the peptides resulting from cutting by elimination of amino acid residues from the N-terminal ends and/or C-terminal ends of SEQ ID No 1, SEQ ID No 2, SEQ ID No 3 or SEQ ID No 4, and the peptides resulting from lengthening by addition of the residues to any of the peptides of SEQ ID No 1, SEQ ID No 2, SEQ ID No 3 or SEQ ID No 4.

In accordance with another preferred embodiment, the peptide is selected from a group that comprises peptide SEQ ID No 1, the peptides with a sequence resulting from elimination of residues of N-terminal and/or C-terminal amino acids from SEQ ID No 1 and the peptides resulting from adding to any of the preceding sequences, the residues of amino acids necessary for protein conjugation.

In another preferred embodiment of the present invention, the peptide is selected from among the group made up by the peptide of SEQ ID No 2, the peptides with a sequence resulting from elimination of residues of N-terminal and/or C-terminal amino acids from SEQ ID No 2 and the peptides resulting from the addition to any of the preceding sequences, the residues of amino acids necessary for protein conjugation.

In another preferred embodiment of the present invention, the peptide is selected from among the group made up by the peptide of SEQ ID No 3, the peptides with a sequence resulting from elimination of residues of N-terminal

and/or C-terminal amino acids from SEQ ID No 3 and the peptides resulting from the addition to any of the preceding sequences, the residues of amino acids necessary for protein conjugation.

5 In another preferred embodiment of the present invention, the peptide is selected from among the group made up by the peptide of SEQ ID No 4, the peptides with a sequence resulting from elimination of residues of N-terminal and/or C-terminal amino acids from SEQ ID No 4 and the peptides resulting from the addition to any of the preceding sequences, the residues of amino  
10 acids necessary for protein conjugation.

In accordance with another embodiment of the present invention, this provides the use of an antibody or an active fragment or derivative of an antibody that specifically recognizes any of the predominant variants of the beta amyloid  
15 peptide, A $\beta$ 40 and A $\beta$ 42 in the preparation of a medicament for the prevention and/or treatment of a disease characterized by the accumulation of amyloid deposits in the brain of a patient.

According to a preferred embodiment of the present invention, the antibody or  
20 an active fragment or derivative of the antibody that specifically recognizes any of the predominant variants of the peptide A $\beta$  is obtained from a peptide selected from a group that consists of SEQ ID No 1, SEQ ID No 2, SEQ ID No 3, SEQ ID No 4, optionally shortened by elimination of the amino acid residues from the N-terminal and/or C-terminal ends, and optionally lengthened by  
25 addition of amino acid residues appropriate for protein conjugation.

In another more preferred embodiment, said antibody or active fragment or antibody derivative is obtained by immunization of mammals or birds with a  
30 peptides selected from a group made up of the peptide of SEQ ID No 1, peptides with a sequence resulting from elimination of N-terminal and C-terminal amino acid residues of SEQ ID No 1 and peptides resulting from addition of the residues of amino acids necessary for protein conjugation to any of the preceding sequences.

35 In another more preferred embodiment, said antibody or active fragment or antibody derivative is obtained by immunization of mammals or birds with a

peptides selected from a group made up of the peptide of SEQ ID No 2,  
 peptides with a sequence resulting from elimination of N-terminal and C-  
 terminal amino acid residues of SEQ ID No 2 and peptides resulting from  
 addition of the residues of amino acids necessary for protein conjugation to any  
 5 of the preceding sequences.

In another more preferred embodiment, said antibody or active fragment or  
 antibody derivative is obtained by immunization of mammals or birds with a  
 peptides selected from a group made up of the peptide of SEQ ID No 3,  
 10 peptides with a sequence resulting from elimination of N-terminal and C-  
 terminal amino acid residues of SEQ ID No 3 and peptides resulting from  
 addition of the residues of amino acids necessary for protein conjugation to any  
 of the preceding sequences.

In another more preferred embodiment, said antibody or active fragment or  
 antibody derivative is obtained by immunization of mammals or birds with a  
 peptides selected from a group made up of the peptide of SEQ ID No 4,  
 peptides with a sequence resulting from elimination of N-terminal and C-  
 terminal amino acid residues of SEQ ID No 4 and peptides resulting from  
 20 addition of the residues of amino acids necessary for protein conjugation to any  
 of the preceding sequences.

In this application, the amino acids are abbreviated using the single-letter codes  
 accepted in the field, as indicated below:

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A = Ala = alanine  
 C = Cys = cysteine  
 D = Asp = aspartic acid  
 E = Glu = glutamic acid  
 F = Phe = phenylalanine  
 G = Gly = glycine  
 H = His = histidine  
 I = Ile = isoleucine  
 K = Lys = lysine  
 L = Leu = leucine  
 M = Met = methionine

N = Asn = asparagine  
 P = Pro = proline  
 Q = Gln = glutamine  
 R = Arg = arginine  
 S = Ser = serine  
 T = Thr = threonine  
 V = Val = valine  
 W = Trp = tryptophane  
 Y = Tyr = tyrosine

The sequences described previously in the present invention, and identified as SEQ ID no 1, SEQ ID no 2, SEQ ID no 3, SEQ ID no 4, correspond to the following amino acid sequences:

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SEQ ID NO 1	LVFFAEDV
SEQ ID NO 2	GLMVGGVV
SEQ ID NO 3	GLMVGGVVIA
SEQ ID NO 4	RHDSGYEVHHQK

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The antibodies obtained from the previous peptides are given the codes SAR-1, SAR-2, SAR-3 and SAR-4 corresponding to those that are shown below:

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SEQ ID NO 1	SAR-2
SEQ ID NO 2	SAR-3
SEQ ID NO 3	SAR-4
SEQ ID NO 4	SAR-1

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The information relating to identification of the peptide sequences, described in the present invention, that accompany the present document in a computer-readable format, is indicated in the list of sequences that is presented along with this document.

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#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Amyloid plaques in brains of Alzheimer patients with antibodies SAR-1, SAR-2, SAR-3 and SAR-4.



Figure 2. Western Blot which shows the specificity of the antibodies. SAR-3 specifically detects the amyloid protein of 40 amino acids (A $\beta$ 40), SAR-4 that of 42 amino acids (A $\beta$ 42) and SAR-1 that of two isoforms, but having greater affinity for the supposedly more neurotoxic A $\beta$ 42. In each lane, the indicated peptide has been loaded (A $\beta$ 40 or A $\beta$ 42) with the specified amounts in nanograms (10, 100, 200 or 500). In the Western blots, it is also seen that the antibodies SAR-3 and SAR-4 detect both monomers (much more abundant) and dimers of the corresponding peptide.

## EXAMPLES

The present invention is illustrated by means of the following examples.

### Example 1. Generation of polyclonal antibodies.

The four polyclonal antibodies against the four peptides conjugated with KLH that were used as immunogen were generated by immunization in New Zealand white rabbits.

Each immunogen was injected into two rabbits, with five injections in each rabbit: the first intradermal injection of the peptide-KLH conjugate in PBS and emulsified in complete Freud adjuvant and four more intramuscular injections, as a booster dose on days 14, 28, 49 and 80, of the same peptide-KLH conjugate in PBS but this time emulsified in incomplete Freud adjuvant, with the blood letting done at 90 days to detect the presence of antibodies.

After collecting blood, the serum was separated and pre-purified by desalination and then the antibodies were purified by affinity in a matrix comprising 1.5 ml of EMD-Epoxy activated material (Merck) to which 5 mg of the corresponding peptide was added. The purified fractions were packed in 0.1% BSA (Sigma) and stored at 4°C, and glycerol 20-50% could be added as a cryoprotector.

### Example 2. WESTERN-BLOT for A $\beta$

#### 1. Electrophoresis

The Laemmli method was used, described in Current Protocols in Molecular Biology, John Wiley and Sons, New York, 1998, modified by improve the separation of small peptides.

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The apparatus used was a Miniprotean 3 from Bio-Rad.

A 15% acrylamide gel was used, mixed with the following components:

STOCK SOLUTIONS	SEPARATING GEL (15%)	STACKING GEL
40% acrylamide	3.75 ml	500 µl
Tris 3 M, pH = 8.45	3.3 ml	250 µl
Glycerol	1.05 ml	-
Water	1.9 ml	4.2 µl
SDS 20%	50 µl	18.6 µl
APS 10%	50 µl	25 µl
TEMED	10 µl	5 µl

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Initial stock solutions of peptide A $\beta$ 40 and 42 of 1 mg/ml were used (dissolved in PBS). The volume necessary was taken of these solutions for each one of the samples and made up to 20 µl with SBLT (SBL + Tris base 2 M). The samples were then boiled for 5 minutes to denature the peptides and eliminate possible proteases.

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The center of the cuvette was filled with cathode buffer and the outside with anode buffer, the composition of these buffers being as follows:

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Anode buffer

24.2 g Tris base (0.2 M final concentration)

Dilute to 1 litre with H<sub>2</sub>O

Adjust of pH 8.9 with concentrated HCl

Store at 4°C for up to 1 month

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Cathode buffer

12.11 g Tris base (0.1 M final concentration)

17.92 g tricine (0.1 M final concentration)

1 g SDS (0.1% final concentration)

Dilute to 1 litre with H<sub>2</sub>O

Do not adjust pH

5 Store at 4°C for up to 1 month

Finally, the samples were loaded into the wells: 20 µl/well. Using the Polypeptide Standard Kaleidoscope from Bio-Rad as a marker, migration started at low voltage (30 V), and then the voltage was raised to 100 V, after  
10 approximately 1 hour of electrophoresis.

## 2.- MEMBRANE TRANSFER

The proteins separated in the gel were transferred to the PVDF membrane by electroblotting. In the transfer booklets the following were placed  
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Black side –sponge- 3 Whatmann papers (or filter papers) –gel- -membrane- -3 Whatmann papers- -sponge- -transparent side.

20 The cuvette was then filled with electroblotting buffer:

Glycine 38 mM

Tris base 50 mM

Methanol 40%

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The transfer was done for 2 hours at 200 mA. During the transfer, the buffer was kept stirring with the magnetic stirrer.

## 3- INCUBATION WITH ANTIBODIES

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The antibodies and the powder milk were dissolved in PBS-t (PBS + 0.5% Tween 20), carrying out the washing with PBS-T also.

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After the transfer, the surface of the membrane was blocked with 5% solution of powder milk for 1 hour with stirring and at room temperature (RT)

After this, the membrane was washed for 2 x 5 minutes at RT.

Then, it was incubated with primary antibody (SAR-1, SAR-2, SAR-3 or SAR-4) for 1 hour at RT at least diluted 1:500 in PBS-T.

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The membrane was washed: 3 x 10 minutes at RT. Then, it was incubated with secondary antibody: goat anti-rabbit-HRP for 1 hour at RT (1:10,000 in all cases).

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The washing of the membrane was repeated once again: 3 x 10 minutes at RT.

#### 4.- DEVELOPMENT

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After the last washing, the membrane was incubated with the solution of the chemoluminescence kit, using the ECL kit+Plus from Pharmacia.

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The membrane was wrapped in cellophane and exposed to double-emulsion film (Hyperfilm MP from Amersham), for different times of between 30 seconds and 2 minutes.

Example 3. Immunohistochemistry with SAR-1, SAR-2, SAR-3 and SAR-4 antibodies in the tissue of human brain.

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The sections of tissue were fixed in paraffin following the following steps:

- a) fixation in neutral formol at 10%
- b) dehydration by successive steps in increasing concentrations of alcohol
- c) passes through xylol and paraffin, this latter step in an oven at 60-62°C.
- d) carrying out of paraffin blocks, which were cut to 4 microns and mounted in slides.

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The sections were then deparaffinized by passing through the following solutions:

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Xylol 100% 10 minutes

Xylol 100% 10 minutes

Ethanol 100% 5 minutes

	Ethanol	100%	5 minutes
	Ethanol	96%	5 minutes
	Ethanol	90%	5 minutes
	Ethanol	70%	5 minutes
5	PBS		5 minutes x 3 times

Afterwards, they were treated in the following way:

- a) 96% formic acid for 3 minutes in a fume cupboard and with stirring
- b) Rapid washing with water
- 10 c) Washing in PBS 2 x 5 minutes
- d) Block of the endogenous peroxidases for 15 minutes in a solution made up of 70 ml of PBS, 30 ml of methanol and 1 ml of H<sub>2</sub>O<sub>2</sub>
- e) Washing in PBS 3 x 5 minutes
- f) Washing in PBS/T (Triton or Tween-20 at 0.5% in PBS) 3 x 5 minutes
- 15 g) Block of the non-specific binding with goat serum (Normal Goat Serum) diluted 10:100 in PBS/T for two hours
- h) Incubation of the primary antibodies all night at 4°C in a moisture chamber:
- 20 Sar-1... Dilution 1:150 in PBS
- Sar-2... Dilution 1:1500 in PBS
- Sar-3... Dilution 1:1500 in PBS
- Sar-4... Dilution 1:2000 in PBS
- 25 i) Washing in PBS/T 3 x 5 minutes
- j) Incubation in secondary antibody (anti-rabbit goat) diluted 1:200 in PBS during 45 minutes
- k) Washing in PBS 4 x 5 minutes
- l) Incubation of ABC (avidin-biotin complex) of Vector Labs at a dilution of
- 30 1:100 in PBS/T for 45 minutes in darkness, keeping these conditions until development was complete
- m) Washing in PBS 3 x 5 minutes
- n) Development in diaminobenzidine (DAB)
- 35 The time was controlled empirically under a stereoscopic microscope. For this, first, a washing was done in a solution of Tris-HCl 0.5 M for 10 minutes with

shaking, to then continue with incubation with a diaminobenzidine substrate (DAB) diluted in Tris-HCl 0.05M and to which is added 0.5  $\mu$ l/ml of H<sub>2</sub>O<sub>2</sub> at 4°C. Once the reaction was finished, three washes were done in PBS at 4°C for 5 minutes each time and then dehydration in ethanol was done at 70%, 90% and 100% for 2 minutes each time, passing through xylol for 4 minutes and a further pass through xylol for 2 minutes, until they were mounted with Eukitt for observation under the microscope.

List of sequences.

NUMBER OF SEQUENCES: 4

INFORMATION ON SEQUENCE 1:

CHARACTERISTICS OF THE SEQUENCE:

LENGTH: 8

TYPE: amino acid

TYPE OF MOLECULE: peptide

SOURCE: Chemical synthesis

SEQUENCE DESCRIPTION:

SEQ ID NO 1

Leu Val Phe Phe Ala Glu Asp Val

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INFORMATION ON SEQUENCE 2:

CHARACTERISTICS OF THE SEQUENCE:

LENGTH: 8

TYPE: amino acid

TYPE OF MOLECULE: peptide

SOURCE: Chemical synthesis

SEQUENCE DESCRIPTION:

SEQ ID NO 1

Gly Leu Met Val Gly Gly Val Val

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## INFORMATION ON SEQUENCE 3:

## CHARACTERISTICS OF THE SEQUENCE:

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LENGTH: 10

TYPE: amino acid

TYPE OF MOLECULE: peptide

SOURCE: Chemical synthesis

## SEQUENCE DESCRIPTION:

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SEQ ID NO 1

Gly Leu Met Val Gly Gly Val Val Ile Ala

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## INFORMATION ON SEQUENCE 4:

## CHARACTERISTICS OF THE SEQUENCE:

LENGTH: 12

TYPE: amino acid

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TYPE OF MOLECULE: peptide

SOURCE: Chemical synthesis

## SEQUENCE DESCRIPTION:

SEQ ID NO 4

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Arg His Asp Ser Gly Tyr Glu Val His His Gln Lys

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